REPORT DOCUMENTATION PAGE WRAIR #93

Form Approved
OMB No. 0704-0188

AD-A277 774

1. AGENCY

3. REPORT TYPE AND DATES COVERED

4. TITLE AND SUBTITLE
Local Immune Response and Protection in the Guinea Pig Keratoconjunctivitis Model following Immunization with Shigella Vaccines

5. FUNDING NUMBERS

6. AUTHOR(S)
Antoinette B. Hartman, Lillian Van De Verg, Hugh H. Collins, Jr., Douglas B. Tang, Natalie O. Bendiuk, David N. Taylor, and Calvin J. Powell

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Walter Reed Army Institute of Research
Washington DC 20307-5100

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command
Ft. Detrick, Frederick, MD 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
APPROVED FOR PUBLIC RELEASE:
DISTRIBUTION UNLIMITED

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

This study used the guinea pig keratoconjunctivitis model to examine the importance of route of administration (mucosal versus parenteral), frequency and timing of immunization (primary versus boosting immunization), and form of antigen given(live attenuated vaccine strain versus O-antigen-protein conjugate) on the production or protective immunity against Shigella infection. Since local immune response to the lipopolysaccharide (LPS) O-antigen of Shigella ssp. is thought to be important for protection against disease, O-antigen-specific antibody-secreting cells (ASC) in the spleen and regional lymph nodes of immunized animals were measured using an ELISPOT assay. Results indicated that protective efficacy was associated with a strong O-antigen-specific ASC response, particularly in the superficial ventral cervical lymph nodes draining the conjunctivae. In naive animals, a strong ASC response in the cervical lymph nodes and protection against challenge were detected only in animals that received a mucosal immunization. Protection in these animals was increased by a boosting mucosal immunization. While parenteral immunization in alone with an O-antigen-protein conjugate vaccine did not protect naive animals against challenge, a combined parenteral-mucosal regimen elicited enhance protection without the addition of a boosting immunization. Although O-antigen-specific serum immunoglobulin A titers were significantly higher in animals receiving a mucosal immunization, there was no apparent correlation between levels of serum antibody and protection against disease.

14. SUBJECT TERMS
Shigella vaccines, immune response, protective efficacy, guinea pig keratoconjunctivitis model.

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

INFECTION AND IMMUNITY, Feb. 1994, p. 412–420
0019-9567/94/$04.00+0

Vol. 62, No. 2

Local Immune Response and Protection in the Guinea Pig Keratoconjunctivitis Model following Immunization with *Shigella* Vaccines

ANTOINETTE B. HARTMAN,[1]* LILLIAN L. VAN DE VERG,[1] HUGH H. COLLINS, JR.,[2] DOUGLAS B. TANG,[3] NATALIE O. BENDIUK,[1] DAVID N. TAYLOR,[1] AND CALVIN J. POWELL[4]

Departments of Enteric Infections,[1] Biostatistics and Math,[3] and Biologics Research,[4] Walter Reed Army Institute of Research, Washington, D.C. 20307-5100, and Univax Biologics, Inc., Rockville, Maryland 20852[2]

Received 15 September 1993/Returned for modification 29 October 1993/Accepted 18 November 1993

This study used the guinea pig keratoconjunctivitis model to examine the importance of route of administration (mucosal versus parenteral), frequency and timing of immunization (primary versus boosting immunization), and form of antigen given (live attenuated vaccine strain versus O-antigen–protein conjugate) on the production of protective immunity against *Shigella* infection. Since local immune response to the lipopolysaccharide (LPS) O-antigen of *Shigella* spp. is thought to be important for protection against disease, O-antigen-specific antibody-secreting cells (ASC) in the spleen and regional lymph nodes of immunized animals were measured by using an ELISPOT assay. Results indicated that protective efficacy was associated with a strong O-antigen-specific ASC response, particularly in the superficial ventral cervical lymph nodes draining the conjunctivae. In naive animals, a strong ASC response in the cervical lymph nodes and protection against challenge were detected only in animals that received a mucosal immunization. Protection in these animals was increased by a boosting mucosal immunization. While parenteral immunization alone with an O-antigen–protein conjugate vaccine did not protect naive animals against challenge, a combined parenteral-mucosal regimen elicited enhanced protection without the addition of a boosting immunization. Although O-antigen-specific serum immunoglobulin A titers were significantly higher in animals receiving a mucosal immunization, there was no apparent correlation between levels of serum antibody and protection against disease.

Shigellae are enteric pathogens that invade the human colonic epithelium and multiply intracellularly, causing bacillary dysentery. Shigellosis is particularly prevalent in developing countries, but it is also found in industrialized countries, especially in institutional settings. It has been estimated that shigellosis is responsible for more than half a million deaths a year, mostly in young children (38), making the development of a safe and effective *Shigella* vaccine an important goal. Epidemiological evidence has indicated that naturally acquired immunity to shigellosis is species and serotype specific (2, 9, 12, 17, 26), and there is also evidence that the presence of serum antibodies against the lipopolysaccharide (LPS) O-antigen are associated with protection against disease (2, 3, 5). Thus, the LPS O-antigen may be a critical antigen in the development of protective immunity against disease.

Local immunity is thought to play an important role in defense against bacterial enteropathogens like shigellae that infect mucosal surfaces (19, 20). After earlier studies demonstrated that parenteral immunization with live or killed shigellae did not protect against infection (11, 18, 37), recent efforts have emphasized the development of orally administered attenuated vaccine strains to induce mucosal immunity to *Shigella* antigens (9, 10, 21, 24–27). Although several candidates have shown promise, the development of a safe and efficacious *Shigella* vaccine has been a difficult task. Therefore, maximizing the protective immunity produced by a vaccine against shigellosis is crucial.

The ability of shigellae to invade the corneal epithelia of guinea pigs and to spread to contiguous cells, causing keratoconjunctivitis, provides a model system to test the virulence of *Shigella* strains and the protective efficacy and immunogenicity of *Shigella* vaccines (16, 36). In the present study, the guinea pig keratoconjunctivitis model was used to examine the effect of route of administration (mucosal versus parenteral), frequency of immunization (primary versus boosting immunization), and form of antigen given (live attenuated vaccine strain versus O-antigen–protein conjugate) on the induction of protective immunity against disease in naive animals and to examine the relationship of the serum and local immune responses generated by immunization to protection. Holmgren et al. (19) proposed that determination of the antibody response against protective antigens at the mucosal site of antigen stimulation may represent a more accurate representation of the local immune response than the measurement of antibodies in body fluids where degradation and half-life of antibody molecules influence the accuracy of analyses. Measurement of antibody-secreting cells (ASC) in regional lymph nodes or mucosal tissues has been used in a variety of systems to determine the local immune response to a specific antigen and has been shown to correlate with levels of secretory immunoglobulin A (secretory IgA) (8, 13, 28, 32). In this study, the local immune response to the LPS O-antigen induced by immunization was determined by measuring the O-antigen-specific ASC in the regional lymph nodes draining the eye, the site of immunization and infection, and in the spleen, Peyer's patches, and other lymph nodes by using the enzyme-linked

* Corresponding author. Mailing address: Department of Enteric Infections, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Building 40, Washington, DC 20307-5100. Phone: (202) 576-3792. Fax: (202) 576-0748.

immunospot (ELISPOT) assay. The serum antibody response to the O-antigen was determined after immunization. In addition, since previous experiments have suggested that parenteral priming followed by mucosal immunization enhances the mucosal response to *Shigella* antigens (22), studies were designed to test whether combination regimens increased the protective efficacy against disease.

MATERIALS AND METHODS

Bacterial strains and vaccines. The virulent strain *Shigella flexneri* serotype 2a 2457T was obtained from the Walter Reed Army Institute of Research collection and used to challenge guinea pigs. When used for challenge, the strain was first streaked on Congo red plates (0.01% Congo red dye [Difco Laboratories, Detroit, Mich.] in Trypticase soy agar (Difco); Congo red-positive colonies were then selected and spread for growth on Trypticase soy agar plates and grown overnight at 37°C. Samples were harvested the next day with 10 ml of phosphate-buffered saline (PBS), and the suspension was used for challenge. The inoculum was determined by colony counts on Trypticase soy agar plates. Vaccine strain EcSf2a-2 is an *aroD*-deleted *Escherichia coli-S. flexneri* 2a hybrid vaccine carrying *Shigella* chromosomal and plasmid genes in an *E. coli* background (27). The vaccine lot used in this study was prepared by the Salk Institute (Swift Water, Pa.) as described previously (16). For immunization, lyophilized bacteria were rehydrated in 5 ml of sterile distilled water and kept on ice for 30 min with repeated swirling. The polysaccharide-protein conjugate vaccines (3, 34, 35) used in this study were a generous gift of John B. Robbins, National Institute of Child Health and Human Development, Bethesda, Md. The vaccine directed against homologous shigellae consists of the O-specific side chain of *S. flexneri* 2a conjugated to *Pseudomonas aeruginosa* exoprotein A (50 μg/ml). The heterologous vaccine used in this study consists of the O-specific side chain of *Shigella sonnei* conjugated to *P. aeruginosa* exoprotein A (50 μg/ml). The nonshigellae control used in this study was *Pneumococcus* type 6A polysaccharide conjugated to tetanus toxoid (100 μg/ml).

Immunization regimens. Vaccination regimens were varied by route of administration and the frequency and timing of immunization, as shown in Table 1. In each immunization group, there were 4 to 10 animals, as shown in Tables 2 to 4. The hybrid *E. coli-S. flexneri* 2a vaccine strain EcSf2a-2 (27) was administered by a mucosal route, i.e., ocular inoculation, by using a primary immunization only, as in protocol M, or primary and boosting immunizations, as in protocol M-B. A polysaccharide-protein conjugate vaccine consisting of *S. flexneri* 2a O-antigen conjugated to *P. aeruginosa* exoprotein A (3, 34, 35) was administered intraperitoneally (i.p.) by using aluminum hydroxide as the adjuvant, either in a single dose (protocol IP) or with two boosting doses (protocol IP-B). Two combination parenteral-mucosal regimens were tested (protocols IPM and IPM-B, which included a boosting dose of EcSf2a-2). Nonimmunized animals were used to determine background readings (protocol NC) and as controls in protection experiments. Immunization with an unrelated polysaccharide conjugate (protocol IPC), *Pneumococcus* type 6A polysaccharide conjugated to tetanus toxoid, was used as a control to ensure that nonspecific immunity induced by administration of a polysaccharide conjugate was not responsible for protection against virulent challenge or for the local immune response to *Shigella* O-antigen. To determine whether the priming influence of the parenterally administered O-antigen–protein conjugate was the result of serotype-specific stimulation, a heterologous O-antigen conjugate in which *S. sonnei* O-antigen, which is not cross-reactive with *S. flexneri* O-antigen, was conjugated to exoprotein A was used in the initial parenteral immunization (protocol IPM-son). For additional studies on the protective efficacy of the combined parenteral-mucosal regimen, immunizations were done by using a subcutaneous (s.c.) route of administration for the parenteral immunization. The *S. flexneri* 2a polysaccharide-protein conjugate vaccine was administered twice subcutaneously (protocol IP-B-SC). Three combination regimens were tested (protocols IPM-SC, IPM-B-SC, and MIP-SC). In MIP-SC, the primary dose was given mucosally, and the boosting dose was given subcutaneously. A regimen using primary and boosting mucosal doses of EcSf2a-2 [protocol M-B(a)] was also included in this experimental group.

TABLE 1. Immunization protocols

Protocol	Primary immunization		First booster immunization		Second booster immunization	
	Vaccine route[a]	Days	Vaccine route	Days	Vaccine route	Days
NC	Nonimmunized					
M	ES-OC	0, 2, 4				
M-B	ES-OC	0, 2, 4	ES-OC	14, 15		
IPM	2a-IP	0				
	ES-OC	2, 4				
IPM-son	son-IP	0				
	ES-OC	2, 4				
IPM-B	2a-IP	0	ES-OC	14, 15		
	ES-OC	2, 4				
IP	2a-IP	0				
IP-B	2a-IP	0	2a-IP	14	2a-IP	28
IPC	Pn-IP	0	Pn-IP	14	Pn-IP	28
M-B(a)	ES-OC	0, 2, 4	ES-OC	14		
IPM-SC	2a-SC	0				
	ES-OC	2, 4				
IPM-B-SC	2a-SC	0	ES-OC	14		
	ES-OC	2, 4				
IP-SC	2a-SC	0				
IP-B-SC	2a-SC	0	2a-SC	14		
MIP-SC	ES-OC	0, 2	2a-SC	14		

[a] ES-OC, hybrid *E. coli-S. flexneri* 2a vaccine strain EcSf2a-2 inoculated ocularly from reconstituted lyophilized vials in doses of 2×10^8 to 4×10^8 organisms per eye; 2a-IP, *S. flexneri* 2a O-polysaccharide conjugated to *P. aeruginosa* exoprotein A given to each animal i.p. in a dose of 0.25 ml of a 50-μg/ml preparation mixed with 0.25 ml of aluminum hydroxide; 2a-SC, *S. flexneri* 2a O-polysaccharide-protein conjugate given to each animal s.c. in a dose of 0.25 ml of a 50-μg/ml preparation; son-IP, *S. sonnei* O-polysaccharide conjugated to *P. aeruginosa* exoprotein A given to each animal i.p. in a dose of 0.25 ml of a 50-μg/ml preparation mixed with 0.25 ml of aluminum hydroxide; Pn-IP, *Pneumococcus* type 6A polysaccharide conjugated to tetanus toxoid given to each animal i.p. in a dose of 0.125 ml of a 100-μg/ml preparation mixed with 0.125 ml of normal saline and 0.25 ml of aluminum hydroxide.

Immunization of animals. Ocular immunizations were done as described previously (16). In the case of the live EcSf2a-2 vaccine, bacteria were reconstituted as described in "Bacterial strains and vaccines" above, and 0.05 ml was inoculated into each eye of the animals. For each immunization, the inoculum was determined by colony counts on Trypticase soy agar plates; the average dose was 2×10^8 to 4×10^8 CFU per eye. For i.p. administration of the polysaccharide-protein conjugate vaccines, the vaccine was thoroughly mixed with an equal volume of aluminum hydroxide suspension (Imject Alum; Pierce Chemical Co., Rockford, Ill.) prior to injection. The dose of the *S. flexneri* 2a and the *S. sonnei* conjugate vaccines was 0.25 ml of a 50-μg/ml solution, which is five times the dose used for immunogenicity studies in mice (3); the dose of the *Pneumo-*

coccus vaccine used as a control was 0.125 ml of a 100-μg/ml solution. s.c. administration did not use the alum adjuvant; at least four immunizations were given under the front legs and on the back of the animal at the same doses used for the i.p. immunizations.

Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for Care and Use of Laboratory Animals* (26a).

Protection studies. Fourteen days after the last immunization, the animals were challenged with virulent *S. flexneri* 2a strain 2457T at a dose of 4×10^8 to 5×10^8 CFU per eye, as determined by colony count of the inoculum. After challenge, the animals were examined for 7 days for development of keratoconjunctivitis. The degree of keratoconjunctivitis was rated on the basis of time of development and severity of symptoms (16). The severity of infection in the eyes of animals was rated after challenge by the following four-point scale: 0, no disease or mild irritation; 1, mild keratoconjunctivitis or late development and/or rapid clearing; 2, keratoconjunctivitis, but no purulence; 3, fully developed keratoconjunctivitis, with purulence. A rating for each animal was determined by the highest eye rating. Protection was defined as follows: full protection, no disease or mild irritation (score of 0); partial protection, mild keratoconjunctivitis or late development and/or rapid clearing (score of 1); combined protection, full or partial protection (score of 0 or 1).

Preparation of cells for ELISPOT assay. Animals were sacrificed by injection with sodium pentobarbital, and the spleens, regional lymph nodes (superficial ventral cervical lymph nodes [SVCLN], mandibular nodes [MDLN], and mesenteric nodes [MSLN]), and Peyer's patches (PP) were harvested. After the lymph nodes were teased to release the lymphocytes, the cell suspension was put through a sterile screen to remove debris and washed once with RPMI 1640 medium (GIBCO Laboratories, Gaithersburg, Md.) containing 15 μg of gentamicin (GIBCO) per ml. The erythrocytes in a single-cell suspension of the spleens were lysed with erythrocyte lysing buffer (Sigma Chemical Co., St. Louis, Mo.) and washed an additional time with 40 ml of RPMI 1640 medium. Cells were counted and suspended in RPMI 1640 medium with 10% fetal calf serum, 2 mmol of L-glutamine per liter, and 15 μg of gentamicin per ml at 2.5×10^6 cells per ml.

ELISPOT assay. The O-antigen-specific ASC response of the animals to immunization and challenge was determined by using a modification of the ELISPOT assay based on the method of Czerkinsky et al. (7). Briefly, each well of 96-well microtiter plates (Nunc-Immuno Maxisorb plates; Nunc, Roskilde, Denmark) was coated with 1 μg of *S. flexneri* 2a LPS (prepared by the method of Westphal and Jann [41]) in 20 mM Na_2CO_3 buffer (pH 9.6) or with carbonate buffer alone. After being washed once with PBS, the plates were blocked with 5% fetal calf serum in PBS for 1 h and washed with PBS prior to use. Prepared cells were dispensed at 100 μl per well, and the plates were incubated for 4 h at 37°C in a humidified CO_2 incubator. After incubation, the plates were washed four times with PBS–0.05% Tween 20 (PBS-Tween). 100 μl of anti-guinea pig IgG, IgA, or IgM (ICN Laboratories) per well was added (anti-guinea pig IgG, 1:1,200 dilution in casein solution, consisting of 20 g of casein and 2.0 g of sodium azide in PBS per liter [pH 7.2 to 7.4]; anti-guinea pig IgA and IgM, 1:800 dilution), and the plates were incubated at 4°C overnight. After the plates were washed with PBS-Tween, 100 μl of goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) at a 1:1,200 dilution in casein was added to each well, and the plates were incubated for 2 h at 37°C. After the plates were washed with PBS-Tween, to each well was added 100 μl of a melted agarose-substrate overlay (0.7% type I, low-EEO [electroendosmosis] agarose [Sigma] in 100 ml of barbital buffer [pH 9.6] [Sigma] containing 0.4 ml of 1 M $MgCl_2$ and 2 ml of BCIP [5 mg of *p*-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate *toluidinium* per ml [Sigma] in 1 ml of *N,N*-dimethyl formamide]). Antigen-specific ASC were visualized as blue spots, which were counted with a stereomicroscope and recorded as ASC per 10^6 cells.

ELISA. An enzyme-linked immunosorbent assay was used to quantitate the serum antibody response to *S. flexneri* 2a LPS as described previously (16) but with modifications to measure all isotypes. The LPS antigen was diluted to a concentration of 10 μg/ml in carbonate coating buffer (described above), the wells of polyvinyl microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated with 50 μl of LPS solution or carbonate buffer alone, and the plates were incubated overnight at 4°C. After being washed, the plates were blocked with casein solution for 1 h at 37°C. After washing the plates with PBS-Tween, guinea pig sera, serially diluted twofold in casein buffer, were added to the wells, and the plates were incubated for 2 h at 37°C. After washing the plates, 50 μl of anti-guinea pig IgG, IgA, or IgM (ICN Laboratories) in the dilutions used in the ELISPOT assay was added to each well, and the plates were incubated for 2 h at 37°C. After the plates were washed, 50 μl of a 1:1,200 dilution in casein of goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Laboratories) was added to each well. After a 2-h incubation at 37°C, the plates were washed with PBS-Tween and 100 μl of the substrate (a 1-mg/ml concentration of *p*-nitrophenyl phosphate in 1 M diethanolamine buffer, [pH 9.8], containing 0.5 mM $MgCl_2$) was added to each well. Optical density was read at 405 nm. Endpoint titers were defined as the last dilution having an optical density of 0.1 or greater above that of the background wells (carbonate buffer only). Titers of sera obtained from the animals prior to immunization were used to determine background titers; these values were <25 for IgA and IgM and <50 for IgG in all animals.

Statistical analysis. To determine the magnitude and timing of the local response to critical *Shigella* antigens, the ASC counts for the SVCLN, spleen, PP, and other nodes (MSLN and MDLN) were plotted for each animal. Similar plots were constructed for each immunization group for ASC data obtained at 13 days postimmunization ($n = 2$ in each group) and 7 to 8 days postinfection ($n = 2$ in each group). Geometric mean serum titers (± standard error) were used to summarize the serum immune response (IgG, IgA, and IgM) obtained at 13 days postimmunization ($n = 4$ in each vaccine group). Frequency distributions of the infection severity rankings and corresponding protection rates were used to summarize protection data from challenge experiments ($n = 4$ to 8 for each group). Two-way analysis of variance (ANOVA) was used as part of the overall data analysis strategy to assess the differences in outcome measures due to the effects of (i) route of administration (M, IP, IPM), (ii) boosting dose (presence or absence), and (iii) the interaction of route and booster (33). Serum titer data were log transformed (base 10) in all analyses. In the case of the infection severity ratings (ordinal data), this corresponds to the rank-transform method (6). When overall differences (by ANOVA) were significant ($P < 0.05$), specific comparisons (by ANOVA mean square error) were made (e.g., all mucosal [M, M-B, IPM, and IPM-B] versus parenteral only [IP and IP-B]). The unpaired *t* test (ASC or log titer data) or Wilcoxon rank sum test (severity ranking) was used to compare two groups (33). All *P* values for comparing two groups are two

sided. The Minitab software system (version 8.2; Minitab, Inc., State College, Pa.) was used to carry out all statistical analyses.

RESULTS

Measurement of O-antigen-specific ASC in the spleen and regional lymph nodes of infected animals. To establish that the guinea pig keratoconjunctivitis model can be used to measure local immune response to critical *Shigella* antigens induced by infection and to examine the timing of this response, an ELISPOT assay was used to detect *S. flexneri* 2a O-antigen-specific ASC in the spleen, PP, and regional lymph nodes of guinea pigs ocularly infected by *S. flexneri* 2a strain 2457T. The lymph nodes examined were the MDLN, MSLN, and SVCLN, which drain the eye and other head regions.

Animals were infected with strain 2457T, and O-antigen-specific IgG-, IgA-, and IgM-ASC were measured in the spleen, PP, and regional lymph nodes in one or two animals for each time point for 0 to 44 days postinfection (Fig. 1). The spleen, PP, and regional lymph nodes from two uninfected animals were examined on day 0 for background counts and were negative for specific ASC. After infection, the O-antigen-specific ASC response for all three isotypes peaked around 12 days, dropped by 15 days, and waned by 37 to 44 days postinfection, when only a few specific ASC were observed.

As shown in Fig. 1, the majority of O-antigen-specific ASC were located in the SVCLN and the spleen 8 to 12 days postinfection, with the number being at least twofold higher in the SVCLN. By days 15, 18, and 28, the numbers of O-antigen-specific ASC were about equal in the spleen and SVCLN. At all time points, the MSLN and PP, which are distal to the site of infection, contained only a few specific ASC and are presented combined with the MDLN as "other" in Fig. 1.

Local immune response to *S. flexneri* 2a O-antigen after immunization. The local immune response was examined in animals immunized with and without a boosting dose for each route of administration (M and M-B, IPM and IPM-B, IP and IP-B), in nonimmunized animals (NC), and in animals immunized with the unrelated polysaccharide conjugate vaccine (IPC). O-antigen-specific IgG-, IgA-, and IgM-ASC were measured in the spleen, SVCLN, MDLN, MSLN, and PP 13 days after the last immunization (1 day prior to challenge) in animals from each protocol group ($n = 2$), as shown in Fig. 2. Because MDLN, MSLN, and PP showed only scattered specific ASC, the ASC from these sites are shown combined under the designation "other." Although there was some variation within each immunization group, clear patterns of ASC response emerge from the data. A two-way ANOVA (route [M, IPM, or IP] by boosting dose [presence or absence]) indicated significant differences in mean ASC counts between routes (IgG, $P = 0.047$; IgA, $P = 0.024$; IgM, $P = 0.014$). There was no detectable booster effect and no evidence that booster effects depended on route (no interaction). In animals receiving only parenteral immunizations (IP and IP-B), few O-antigen-specific ASC were detected in the animals receiving only one immunization (IP), while specific IgG-ASC were detected in the spleens of animals receiving three immunizations (IP-B). There was little O-antigen-specific IgA-ASC response in animals from either the IP or IP-B group, and no specific ASC were found in the SVCLN. In contrast, animals receiving at least one mucosal immunization (M, M-B, IPM, and IPM-B) had significantly ($P < 0.001$) higher numbers of O-antigen-specific ASC of all isotypes than those receiving only parenteral immunizations, and specific ASC were found in the SVCLN as well as in the spleens of these animals. These results demonstrate that O-antigen-specific ASC were detected in the lymph nodes draining the site of immunization (i.e., SVCLN) only if there was a mucosal immunization. Average ASC counts for the IPM group were somewhat higher than those for the M groups (M or M-B), and the combined regimens (IPM and IPM-B) had a larger proportion of O-antigen-specific IgM-ASC than any of the other groups, suggesting enhanced activation of antigen-specific virgin B cells. Average IgG- and IgA-ASC responses were somewhat increased when boosting regimens (M-B and IPM-B) were administered. Immunization with the *Pneumococcus* polysaccharide conjugate (IPC) produced no detectable O-antigen-specific ASC.

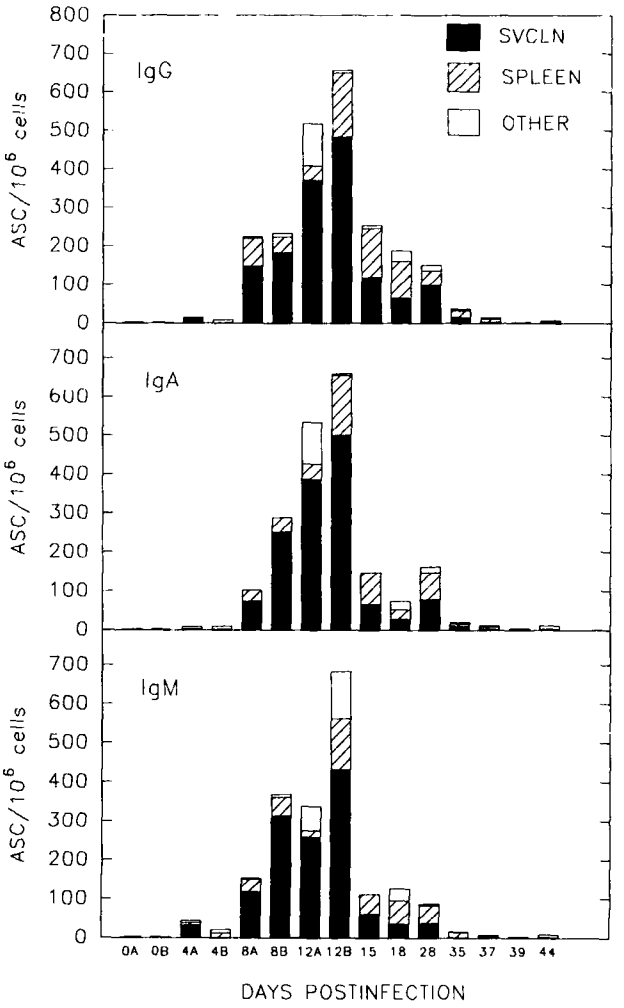

FIG. 1. Number and distribution of O-antigen-specific ASC per 10^6 cells found in the regional lymph nodes, Peyer's patches, and spleen after ocular infection of guinea pigs with *S. flexneri* 2a strain 2457T. O-antigen-specific IgG-, IgA-, and IgM-ASC detected in the spleen, Peyer's patches, and regional lymph nodes were determined for each animal. Background counts were determined by examining ASC in uninfected animals (animals 0A and 0B); these counts were negative. Animals were examined on days 4 (animals 4A and 4B), 8 (animals 8A and 8B), 12 (animals 12A and 12B), 15, 18, 28, 35, 37, 39, and 44 postinfection. For each animal, ASC counts were determined for the SVCLN, the spleen, and "other" sites (MSLN, MDLN, and PP).

Protective efficacy after immunization. Fourteen days after the last immunization, the animals were challenged with virulent *S. flexneri* 2a strain 2457T. The results of the challenge

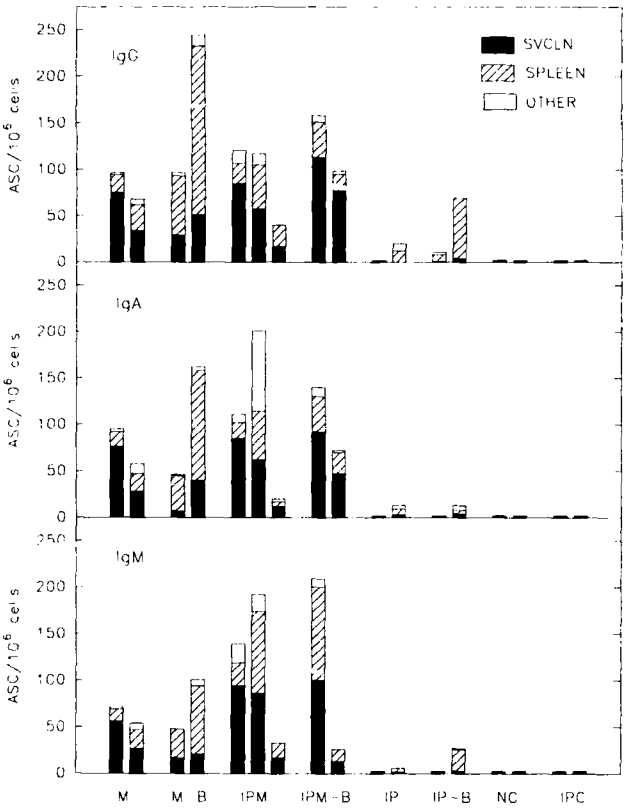

FIG. 2. O-antigen-specific ASC counts per 10^6 cells 13 days postimmunization. Two (three in the case of the IPM group) animals from each immunization group (described in Table 1) were analyzed. For each animal, positive IgG-, IgA-, and IgM-ASC counts were determined for the SVCLN, the spleen, and "other" sites (MSLN, MDLN, and PP).

studies, assessed by the rating of the severity of keratoconjunctivitis, are summarized in Table 2. Statistically significant reductions in severity from that of nonvaccinated animals (NC) were obtained in all cases when the vaccine regimen contained a mucosal immunization with the EcSf2a-2 vaccine (either M, M-B, IPM, or IPM-B compared with NC; maximum P value, 0.02; Wilcoxon rank sum test). When the parenterally administered *S. flexneri* O-antigen conjugate vaccine was given alone (IP) or boosted (IP-B) or when the unrelated polysaccharide conjugate vaccine (IPC) was given, infection severity was essentially identical to that seen in nonimmunized control animals (NC). By using a two-way ANOVA, the overall effect of route of administration (M, IP, or IPM) on protection rates was highly significant ($P < 0.0001$). Although the overall difference between boosted and nonboosted protocols was not significant ($P = 0.083$), there was a significant interaction effect ($P = 0.040$) due to the booster effect seen only in the mucosal group (M, M-B), i.e., a boosting dose in the mucosal group (M-B) significantly increased protection over that of the nonboosted group M (M versus M-B, $P = 0.031$). Notably, there was no significant difference ($P = 0.827$; Wilcoxon rank sum test) between the protection achieved with the nonboosted combined regimen (IPM) and that obtained with the boosted mucosal regimen (M-B), and both IPM and M-B regimens gave significantly greater protection than the M immunization regimen alone (IPM versus M, $P = 0.036$; M-B versus M, $P = 0.031$). Thus, the combined regimen (IPM) achieved protection comparable to that obtained with the M-B regimen without the necessity of a boosting immunization. The addition of a boosting dose to the combination regimen (IPM-B) did not significantly increase protection over that seen in animals receiving the IPM regimen ($P = 0.41$).

TABLE 2. Vaccine protection against virulent challenge[a]

Protocol[b]	No. of animals with severity rating of[c]:				Protection rate (no. of animals protected/total no.)[d]		
	0	1	2	3	Full	Partial	Combined
M	0	3	1	2	0/6 (0)[e]	3/6 (50)	3/6 (50)
IPM	2	3	0	0	2/5 (40)	3/5 (60)	5/5 (100)
IP	0	0	0	4	0/4 (0)	0/4 (0)	0/4 (0)
NC	0	0	0	6	0/6 (0)	0/6 (0)	0/6 (0)
M-B	2	4	0	0	2/6 (33)	4/6 (67)	6/6 (100)
IPM-B	1	5	0	0	1/6 (17)	5/6 (83)	6/6 (100)
NC	0	0	0	5	0/5 (0)	0/5 (0)	0/5 (0)
IP-B	0	1	0	7	0/8 (0)	1/8 (13)	1/8 (13)
IPC	0	0	0	6	0/6 (0)	0/6 (0)	0/6 (0)
NC	0	0	0	6	0/6 (0)	0/6 (0)	0/6 (0)

[a] Challenge was made 2 weeks after the last immunization with 5×10^5 CFU of *S. flexneri* 2a strain 2457T in each eye.

[b] Protocols are described in Table 1.

[c] Severity ratings: 0, no disease or mild irritation; 1, mild keratoconjunctivitis or late development and/or rapid clearing; 2, keratoconjunctivitis, but no purulence; 3, fully developed keratoconjunctivitis, with purulence.

[d] Protection was defined as follows: full, no disease or mild irritation (score of 0); partial, mild keratoconjunctivitis or late development and/or rapid clearing (score of 1); combined, full or partial protection (score of 0 or 1).

[e] Values in parentheses are percentages.

Homologous priming needed in combination regimen for enhanced protective efficacy. To determine whether the priming influence of the parenterally administered O-antigen–protein conjugate was the result of serotype-specific stimulation, a heterologous *S. sonnei* O-antigen conjugate was used in the initial parenteral immunization (IPM-son). The protective efficacies and local immune responses were compared between these animals and animals immunized by regimen IPM by using the homologous O-antigen conjugate (Table 3). Animals from the IPM-son group did not produce an ASC response comparable to that found in the IPM animals (Table 3). Although the infection severity scores in the heterologous group (IPM-son) were significantly less than those of nonimmunized animals ($P = 0.016$; Wilcoxon rank sum test), they were significantly greater than those of the homologous group (i.e., IPM; $P = 0.009$). The corresponding observed protection rates were 38% (3 of 8 animals) for the IPM-son group and 100% (8 of 8 animals) for the IPM group, indicating that homologous priming is necessary for the enhanced protective efficacy observed in the IPM animals.

Combined subcutaneous-mucosal immunizations produce enhanced protective efficacy. To further explore the enhanced protective efficacy observed in the combined parenteral-mucosal immunization regimen, additional experiments using three combined subcutaneous-mucosal regimens (IPM-SC, IPM-B-SC, and MIP-SC; Table 1) were performed. Fourteen days after the last immunization, animals were challenged with the virulent *S. flexneri* 2a strain 2457T. The results of this challenge, shown in Table 4, indicated that significantly greater protection against virulent challenge than that seen in control animals (NC) was observed for all of the combined regimens (IPM-SC, $P = 0.013$; IPM-B-SC, $P = 0.03$; MIP-SC, $P = 0.047$) and for M-B(a) ($P = 0.006$; Wilcoxon rank sum test), in which animals received primary and boosting doses of EcSf2a-2. No

TABLE 3. Protective efficacy[a] and local immune response[b] after homologous and heterologous priming in combination regimens

Protocol[c]	No. of animals with severity rating of:				Protection rate (no. of animals protected/total no.)			No. of ASC/10^6 cells		
	0	1	2	3	Full	Partial	Combined	IgG	IgA	IgM
IPM	1	7	0	0	1/8 (13)[d]	7/8 (88)	8/8 (100)	86	80	116
IPM-son	0	3	3	2	0/8 (0)	3/8 (38)	3/8 (38)	15	11	23
NC	0	0	0	4	0/4 (0)	0/4 (0)	0/4 (0)			

[a] Animals were challenged 2 weeks after the last immunization with 5×10^5 CFU of *S. flexneri* 2a strain 2457T in each eye. The severity ratings of animals and protection levels are described in Table 2, footnotes *c* and *d*.

[b] Average number of O-antigen-specific ASC per 10^6 cells from the spleen, SVCLN, PP, MDLN, and MSLN 13 days postimmunization for three (IPM) and two (IPM-son) animals.

[c] Protocols are described in Table 1.

[d] Values in parentheses are percentages.

protection against virulent challenge was observed in the group immunized twice with the protein-polysaccharide conjugate vaccine (IP-B-SC). Protection in animals immunized by the IPM-SC regimen was comparable to that observed in animals immunized by the M-B(a) regimen. The results of the experiments shown in Tables 2 and 4 show that a primary combined parenteral-mucosal immunization regimen using either the i.p. or s.c. route for the parenteral immunization produces enhanced protection against challenge without the addition of a boosting immunization. The MIP-SC regimen, although producing significant protection over that observed in the control animals, did not produce the enhanced protection observed in the IPM-SC group (MIP-SC, 50%; IPM-SC, 100%). These results indicate the importance of timing and order (i.e., mucosal and then parenteral or parenteral and then mucosal) of the immunizations in the combined regimens.

Local immune response to *S. flexneri* 2a O-antigen after challenge. Two animals from each immunization regimen were sacrificed 7 or 8 days after challenge with virulent *S. flexneri* 2a, and the numbers of O-antigen-specific ASC in the spleen, PP, and regional lymph nodes were determined (Fig. 3). The largest numbers of postchallenge O-antigen-specific ASC were found in the animals which had been immunized three times i.p. with the *S. flexneri* 2a polysaccharide-protein conjugate vaccine (IP-B). Animals receiving the IP-B regimen had significantly higher numbers of specific IgG-ASC than those receiving the other boosting regimens (IP-B versus M-B, $P = 0.027$; IP-B versus IPM-B, $P = 0.0052$). In fact, the IgG-ASC count for the IP-B animals was threefold higher than that found in the M-B animals (average IgG-ASC count, 1,488 versus 458, respectively) and although the differences in specific IgA- and IgM-ASC counts were not significant, these differences were also about threefold higher than those found in the M-B group (average IgA-ASC count, 1,081 versus 348; average IgM-ASC count, 1,200 versus 233). In addition, more than 50% of the postchallenge O-antigen-specific ASC were located in the SVCLN. These results suggest that this conjugate vaccine successfully primed the immune system, but since local priming at the site of infection did not occur prior to challenge, the secondary response seen after infection was too late to provide protection. The second highest ASC counts

TABLE 4. Vaccine efficacy against virulent challenge[a] in combined regimens using subcutaneous-mucosal immunization

Protocol[b]	No. of animals with severity rating of:				Protection rate (no. of animals protected/total no.)		
	0	1	2	3	Full	Partial	Combined
IP-SC	0	1	1	4	0/6 (0)[c]	1/6 (17)	1/6 (17)
IPM-SC	2	2	0	0	2/4 (50)	2/4 (50)	4/4 (100)
NC	0	0	0	4	0/4 (0)	0/4 (0)	0/4 (0)
M-B(a)	1	4	1	0	1/6 (17)	4/6 (67)	5/6 (83)
IPM-B-SC	2	1	1	0	2/4 (50)	1/4 (25)	3/4 (75)
IP-B-SC	0	0	0	6	0/6 (0)	0/6 (0)	0/6 (0)
MIP-SC	1	1	1	1	1/4 (25)	1/4 (25)	2/4 (50)
NC	0	0	0	4	0/4 (0)	0/4 (0)	0/4 (0)

[a] Animals were challenged 2 weeks after the last immunization with 5×10^5 CFU of *S. flexneri* 2a strain 2457T per ml per eye. Severity ratings and protection levels are described in Table 2; footnotes *c* and *d*.

[b] Protocols are described in Table 1.

[c] Values in parentheses are percentages.

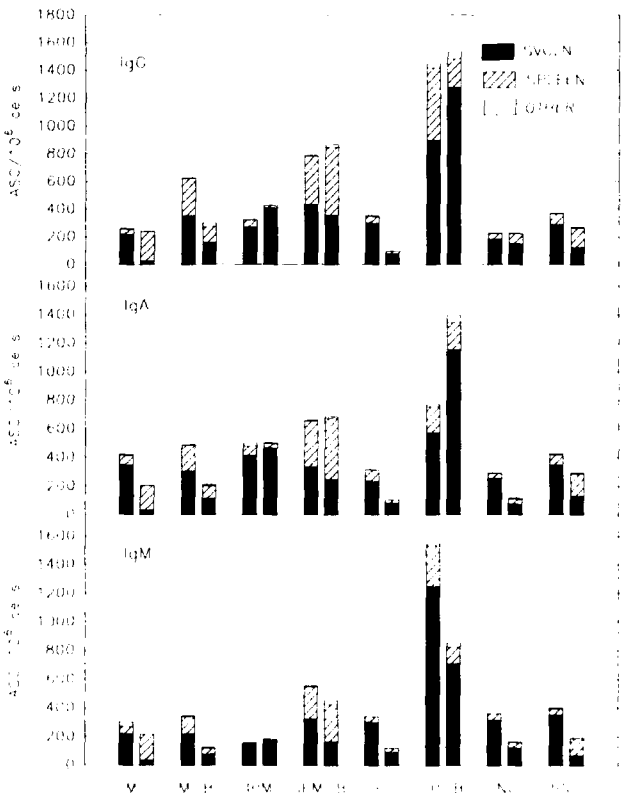

FIG. 3. O-antigen-specific ASC counts per 10^6 cells 8 days postinfection. Two animals from each immunization group (described in Table 1) were analyzed. For each animal, positive IgG-, IgA-, and IgM ASC counts were determined for the SVCLN, the spleen, and "other" sites (MSLN, MDLN, and PP).

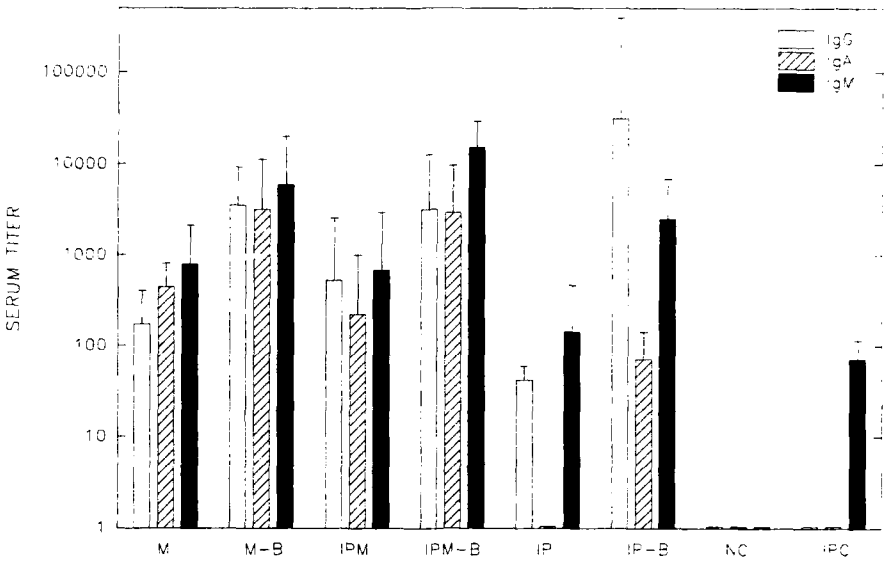

FIG. 4. Serum antibody titers against *S. flexneri* 2a O-antigen 13 days after immunization. Four animals from each immunization group (described in Table 1) were analyzed. The geometric mean titers for serum IgG, IgA, and IgM are shown. Background titers were determined from preimmunization bleeds for each animal (<50 for IgG; <25 for IgA and IgM) and used to calculate endpoint titers as described in Materials and Methods. Standard deviations of the means are indicated.

occurred in animals immunized by protocol IPM-B (average IgG-ASC, IgA-ASC, and IgM-ASC counts were 848, 671, and 502, respectively), suggesting that the parenteral immunization in this group might have increased the priming response. Animals that received only a primary immunization in the vaccination regimen (M, IP, and IPM) did not have a higher proportion of postchallenge O-antigen-specific IgG ASC, as was seen in the animals with a boosting regimen (M-B, IP-B, and IPM-B). Animals that received a combination of parenteral and mucosal immunization with no boost had a higher proportion of O-antigen-specific IgA-ASC than those that received other regimens.

Serum response after immunization. The serum antibody response against *S. flexneri* 2a LPS was examined with and without a boosting dose for each route of administration (M, M-B; IPM, IPM-B; IP, IP-B), for nonimmunized animals (NC), and for animals immunized with the unrelated polysaccharide conjugate vaccine (IPC). The serum IgG, IgA, and IgM geometric mean titers ($n = 4$) are shown in Fig. 4. A two-way ANOVA comparing routes (M, IPM, and IP) with or without boosting indicated a highly significant booster effect for each isotype as evidenced by increased geometric mean titers (minimum of 10-fold) in the boosted regimens (IgG, $P < 0.0005$; IgA, $P < 0.0005$; IgM, $P < 0.0005$). Although a boosting dose increased the serum response for all three routes, the degree of increase varied with route, indicating that there was interaction. Within the groups receiving a mucosal immunization (M, M-B, IPM, and IPM-B), there was no significant difference ($P > 0.05$) for any isotype in geometric mean titers between M and IPM or between M-B and IPM-B. However, animals receiving mucosal immunizations without boosting (M and IPM) had significantly greater geometric mean titers compared with those receiving a single parenteral immunization (IgG, 304 versus 43, $P = 0.040$; IgA, 315 versus 0, $P < 0.0001$; IgM, 740 versus 130, $P = 0.021$). Geometric mean titers for IgA and IgM for boosted mucosal protocols (M-B and IPM-B) were significantly higher than those found in the boosted parenteral protocol IP-B (IgA, 3,089 versus 79, $P = 0.0004$; IgM, 9,526 versus 2,506, $P = 0.07$). Notably, there was no detectable IgA response when a single parenteral immunization was used alone (IP), and in the boosted parenteral regimen (IP-B), the postimmunization IgA titer was minimal (<100). These results suggest an association between immunization route and IgA response. However, there was no apparent correlation between O-antigen-specific serum antibody levels and protection. For example, the IPM animals had serum antibody titers comparable to those found in the M group of animals and lower than those found in the M-B animals, but protection in the IPM animals was equal to that found in the M-B animals and significantly greater than that found in the M animals. There were significantly higher IgG titers in the animals from the IP-B group than in the animals of the boosted mucosal protocols M-B and IPM-B (31,398 versus 3,368, $P = 0.022$), indicating that the polysaccharide-protein conjugate vaccine administered parenterally stimulated antigen-specific IgG production. In the animals receiving the unrelated polysaccharide conjugate vaccine (IPC), there was a small rise in IgM titer (<100), possibly representing stimulation of nonspecific IgM antibodies.

DISCUSSION

Several variables, including the route of administration, the frequency and timing of immunization, and previous exposure to homologous strains, can influence the immune response to protective antigens and thus the protective efficacy afforded by a vaccine (1, 19). In the current study, the guinea pig keratoconjunctivitis model was used to examine the importance of the route of immunization (mucosal versus parenteral), the form of antigen presented (live attenuated strains versus O-antigen–protein conjugate), and the frequency of immunization (primary versus boosting immunizations) on the development of protection against *Shigella* infection in naive animals. The local immune response to the LPS O-antigen induced by immunization or by infection was assessed by detecting the O-antigen-specific ASC in the lymph nodes draining the conjunctivae (i.e., SVCLN) and in the spleen, PP, and other regional lymph nodes.

Our results indicated that, in naive animals, a mucosal immunization via the conjunctival membrane was required for a strong O-antigen-specific ASC response in the SVCLN and for protection against infection. A boosting mucosal vaccination increased protective efficacy. Parenteral immunization alone with an O-antigen subunit vaccine did not stimulate an O-antigen-specific ASC response in the SVCLN even after three immunizations and did not protect against challenge by virulent shigellae. These results emphasize the correlation between the presence of O-antigen-specific ASC in the SVCLN and protection against disease. However, a combined parenteral-mucosal immunization regimen, with either an s.c. or i.p. immunization followed by a mucosal immunization, elicited enhanced protection against *Shigella* challenge without the addition of a boosting immunization.

Previous studies reported a possible correlation between O-antigen-specific serum antibody levels and protection against disease (2, 4), but recent evidence indicated that serum levels may be only a partial reflection of immunity to disease (5). In this study, there was no apparent correlation between levels of serum antibody and protection against disease. For example, the combined mucosal-parenteral regimen (IPM) produced significantly greater protection against disease than the mucosal immunization (M), but animals vaccinated by the IPM regimen did not have significantly higher O-antigen-specific serum antibody titers for any isotype. It should be noted that serum IgA levels were significantly higher in animals receiving a mucosal immunization, but there was no significant correlation between serum IgA levels and protection.

Examination of antigen-specific ASC after oral cholera vaccination in humans showed that the number of anti-cholera toxin B subunit ASC in the peripheral blood mononuclear cells was considerably lower than that in the duodenal mucosa (32). These findings are in agreement with results found in the cynomolgus monkey model of trachoma where the local immune response found in chlamydia-infected monkeys was higher in the regional draining lymph nodes and conjunctivae than in the peripheral blood mononuclear cells and more distal nodes (28). In the guinea pig keratoconjunctivitis model, antigen-specific activated B-lymphocytes can be quantitated in the SVCLN, the regional lymph nodes draining the conjunctivae, as well as in the spleen and more distal nodes, and in this study, the presence of O-antigen-specific ASC responses in the SVCLN was associated with protection. These results demonstrate that measurement of the local immune response in lymphoid tissue associated with the site of immunization may be a better predictor of vaccine efficacy and immunogenicity than measurement in distal sites. For initial vaccine evaluations, such studies can be done more easily in an appropriate animal model.

The enhanced protective efficacy shown by the combined parenteral-mucosal immunization regimen used in this study is in agreement with previous work with *Shigella* spp. (22), *E. coli* heat-labile enterotoxin (23), and cholera toxin (30), showing that parenteral priming followed by a mucosal immunization enhanced mucosal immune responses and, in the cholera toxin study, protection. However, it should be noted that other studies have indicated that parenteral priming had no effect on subsequent immune responses (14) or was suppressive (15, 29, 31), although Pierce did observe that the suppressive effect of parenteral immunization was largely reversed by a subsequent enteric immunization (29). Since it has been suggested that priming for a mucosal response is affected by dosage number and interval as well as immunization route(s) (29), further studies to determine optimum immunization regimens should be made.

It is interesting to note that there was a larger proportion of O-antigen-specific IgM-ASC evident postimmunization in the animals from protocol IPM, suggesting that different compartments of antigen-specific virgin B cells had been stimulated by the two routes of immunization. The use of alum as an adjuvant in the parenteral immunization would also likely increase the magnitude of the priming response. This would potentially result in a larger population of antigen-activated B cells available at the time of challenge than is found when only one route of immunization is used and could contribute to the enhanced mucosal response and protection observed in the animals immunized by the combined parenteral-mucosal regimen. Since a combination parenteral and oral vaccination regimen has the potential to increase local immune responses and enhance protective efficacy with fewer inoculations, additional studies with this combined regimen are planned.

The higher numbers of O-antigen-specific ASC found postchallenge in the IP-B animals suggests that the immune system had been sufficiently primed to produce a large secondary response in the lymph nodes draining the site of infection. However, since there was no local priming prior to infection, migration of O-antigen-specific ASC to the SVCLN was too late to provide protection against disease. It has been shown in other systems that previous exposure to a mucosal pathogen can be stimulated after a parenteral injection. Earlier studies showed that Pakistani women with previous exposure to cholera responded with increased secretory IgA titers in breast milk after a parenteral cholera immunization while no such rise in secretory IgA titers was seen in Swedish women who had no previous exposure to cholera (39, 40). In populations where *Shigella* infection is a common occurrence, it is possible that parenteral immunization can stimulate immunological memory from earlier exposure to homologous strains. This possibility can be tested in this model by parenterally immunizing previously infected animals with the protein-polysaccharide conjugate vaccine or with heat-killed shigellae and measuring the O-antigen-specific ASC response.

The experiments reported here indicate that frequency and route of immunization and the form of the antigen are important variables affecting the development of protection against *Shigella* infection in the guinea pig keratoconjunctivitis model. The results of this study emphasize the importance of mucosal immunization in the development of protective immunity in naive animals. Further experiments using this model can provide valuable information on the ability of immunization regimens to stimulate a local immune response and confer protective immunity, although it should be emphasized that any animal model should be used only as a guide for tests in humans.

ACKNOWLEDGMENTS

We thank John B. Robbins for the generous gift of the polysaccharide-protein conjugate vaccines used in this study. We also thank Sandra Smith-Gill, Richard Warren, and Thomas L. Hale for reading the manuscript and for helpful suggestions.

REFERENCES

1. **Bienenstock, J.** 1988. The nature of immunity at mucosal surfaces—a brief review, p. 9. *In* W. Donachie, F. Griffiths, and J. Stephen (ed.), Bacterial infections of respiratory and gastrointestinal mucosae. IRL Press, Oxford.
2. **Black, R. E., M. M. Levine, M. L. Clements, G. Losonsky, D. Herrington, S. Berman, and S. B. Formal.** 1987. Prevention of shigellosis by a *Salmonella typhi-Shigella sonnei* bivalent vaccine. J. Infect. Dis. **155:**1260–1265.
3. **Chu, C.-Y., B. Liu, D. Watson, S. C. Szu, K. Bryla, J. Shiloach, R. Schneerson, and J. B. Robbins.** 1992. Preparation, characteriza-

tion, and immunogenicity of conjugates composed of the O-specific polysaccharide of *Shigella dysenteriae* type 1 (Shiga's bacillus) bound to tetanus toxoid. Infect. Immun. **40:**245–256.

4. **Cohen, D., M. S. Green, C. Block, T. Rouach, and T. Ofek.** 1988. Serum antibodies to lipopolysaccharide and natural immunity to shigellosis in an Israeli military population. J. Infect. Dis. **157:** 1068–1071.
5. **Cohen, D., M. S. Green, C. Block, R. Slephon, and Y. Lerman.** 1992. Natural immunity to shigellosis in two groups with different previous risks of exposure to *Shigella* is only partly expressed by serum antibodies to lipopolysaccharide. J. Infect. Dis. **165:**785–787.
6. **Conover, W. J.** 1980. Practical nonparametric statistics, 2nd ed. John Wiley & Sons, Inc., New York.
7. **Czerkinsky, C., L.-K. Nilsson, H. Nygren, O. Ouchterlony, and A. Tarkowski.** 1983. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. J. Immunol. Methods **65:**109–121.
8. **Czerkinsky, C., M. W. Russell, N. Lycke, M. Lindblad, and J. Holmgren.** 1989. Oral administration of a streptococcal antigen coupled to cholera toxin B subunit evokes strong antibody responses in salivary glands and extramucosal tissues. Infect. Immun. **57:**1072–1077.
9. **Dupont, H., R. B. Hornick, M. J. Snyder, J. P. Libonatti, S. B. Formal, and E. H. Gangarosa.** 1972. Immunity in shigellosis. II. Protection induced by oral live vaccine or primary infection. J. Infect. Dis. **125:**12–16.
10. **Formal, S. B., and M. M. Levine.** 1984. Shigellosis, p. 167–186. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., New York.
11. **Formal, S. B., R. M. Maenza, S. Austin, and E. H. LaBrec.** 1967. Failure of parenteral vaccines to protect monkeys against experimental shigellosis. Proc. Soc. Exp. Biol. Med. **25:**347–349.
12. **Formal, S. B., E. V. Oaks, R. E. Olsen, M. Wingfield-Eggleston, P. J. Snoy, and J. P. Cogan.** 1991. Effect of prior infection with virulent *Shigella flexneri* 2a on the resistance of monkeys to subsequent infection with *Shigella sonnei*. J. Infect. Dis. **164:**533–537.
13. **Forrest, B. D.** 1988. The identification of an intestinal immune response using peripheral blood lymphocytes. Lancet **i:**81–83.
14. **Forrest, B. D., J. T. LaBrooy, C. E. Dearlove, and D. J. C. Shearman.** 1992. Effect of parenteral immunization on the intestinal immune response to *Salmonella typhi* Ty21a. Infect. Immun. **60:**465–471.
15. **Hamilton, S. R., J. H. Yardley, and G. D. Brown.** 1979. Suppression of local intestinal immunoglobulin A immune response to cholera toxin by subcutaneous administration of cholera toxoids. Infect. Immun. **24:**422–426.
16. **Hartman, A. B., C. J. Powell, C. L. Schultz, E. V. Oaks, and K. H. Eckels.** 1991. Small-animal model to measure efficacy and immunogenicity of *Shigella* vaccine strains. Infect. Immun. **59:**4075–4083.
17. **Herrington, D. A., L. Van De Verg, S. B. Formal, T. L. Hale, B. D. Tall, S. J. Cryz, E. C. Tramont, and M. M. Levine.** 1990. Studies in volunteers to evaluate candidate *Shigella* vaccines: further experience with a bivalent *Salmonella typhi-Shigella sonnei* vaccine and protection conferred by previous *Shigella sonnei* disease. Vaccine **8:**353–357.
18. **Higgins, A. R., T. M. Floyd, and M. A. Kadar.** 1955. Studies in shigellosis. III. A controlled evaluation of a monovalent *Shigella* vaccine in a highly endemic environment. Am. J. Trop. Med. Hyg. **4:**281–288.
19. **Holmgren, J., C. Czerkinsky, J. Lycke, and A.-M. Svennerholm.** 1992. Mucosal immunity: implications for vaccine development. Immunobiology **184:**157–179.
20. **Hone, D., and J. Hackett.** 1989. Vaccination against enteric bacterial diseases. Rev. Infect. Dis. **11:**853–877.
21. **Karnell, A., B. A. D. Stocker, S. Katakure, H. Sweiha, F. P. Reinholt, P. D. Cam, D. D. Trach, and A. A. Lindberg.** 1991. An auxotrophic live oral *Shigella flexneri* vaccine: development and testing. Rev. Infect. Dis. **13**(Suppl. 4)**:**S357–S361.
22. **Keren, D. F., R. A. McDonald, and J. L. Carey.** 1988. Combined parenteral and oral immunization results in an enhanced mucosal immunoglobulin A response to *Shigella flexneri*. Infect. Immun. **56:**910–915.
23. **Klipstein, F. A., R. F. Engert, and J. D. Clements.** 1982. Arousal of mucosal secretory immunoglobulin A antitoxin in rats immunized with *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **37:** 1086–1092.
24. **Lindberg, A. A., A. Karnell, and B. A. D. Stocker.** 1990. Aromatic-dependent *Shigella* strains as live oral vaccines, p. 677–687. *In* G. C. Woodrow and M. M. Levine (ed.), New generation vaccines. Marcel Dekker, Inc., New York.
25. **Lindberg, A. A., A. Karnell, B. A. D. Stocker, S. Katakure, H. Sweiha, and F. P. Reinholt.** 1988. Development of an auxotrophic oral live *Shigella flexneri* vaccine. Vaccine **6:**146–150.
26. **Mel, D. M., B. L. Arsic, B. D. Nikolic, and M. L. Radovanovic.** 1968. Studies on vaccination against bacillary dysentery. IV. Oral immunization with live monotypic and combined vaccines. Bull. WHO **39:**375–380.

26a. **National Institutes of Health.** 1985. Guide for the care and use of laboratory animals. NIH publication 86-23. National Institutes of Health, Bethesda, Md.

27. **Newland, J. W., T. L. Hale, and S. B. Formal.** 1992. Genotypic and phenotypic characterization of an *aroD* deletion-attenuated *Escherichia coli* K12-*Shigella flexneri* hybrid vaccine expressing *S. flexneri* 2a somatic antigen. Vaccine **10:**766–776.
28. **Pal, S., H. R. Taylor, R. B. Huneke, R. A. Prendergast, and J. A. Whittum-Hudson.** 1992. Frequency of antigen-specific B cells during experimental ocular *Chlamydia trachomatis* infection. Infect. Immun. **60:**5294–5297.
29. **Pierce, N. F.** 1984. Induction of optimal mucosal antibody responses: effects of age, immunization route(s), and dosing schedule in rats. Infect. Immun. **43:**341–346.
30. **Pierce, N. F., W. C. Cray, and B. K. Sircar.** 1978. Induction of a mucosal antitoxin response and its role in immunity to experimental canine cholera. Infect. Immun. **21:**185–193.
31. **Pierce, N. F., and F. T. Kostner.** 1980. Priming and suppression of the intestinal immune response to cholera toxoid toxin by parenteral toxoid in rats. J. Immunol. **124:**307–311.
32. **Quilding, M., I. Nordstrom, A. Kilander, G. Andersson, L. A. Hanson, J. Holmgren, and C. Czerkinsky.** 1991. Intestinal immune responses in humans. J. Clin. Invest. **88:**143–148.
33. **Remington, R. D., and M. A. Schork.** 1985. Statistics with applications to the biological and health sciences, 2nd ed. Prentice-Hall, Inc., Englewood Cliffs, N.J.
34. **Robbins, J. B., C. Chu, D. C. Watson, S. C. Szu, E. M. Daniels, C. U. Lowe, and R. Schneerson.** 1991. O-specific side-chain toxin-protein conjugates as parenteral vaccines for the prevention of shigellosis and related diseases. Rev. Infect. Dis. **13**(Suppl. 4)**:**S362–S365.
35. **Robbins, J. B., and R. Schneerson.** 1990. Polysaccharide-protein conjugates: a new generation of vaccines. J. Infect. Dis. **161:**821–832.
36. **Sereny, B.** 1957. Experimental keratoconjunctivitis shigellosa. Acta Microbiol. Acad. Sci. Hung. **4:**367–376.
37. **Shaugnessy, H. J., R. C. Olsson, K. Bass, F. Friewer, and S. O. Levinson.** 1946. Experimental human bacillary dysentery: polyvalent dysentery vaccine in its prevention. JAMA **132:**362–368.
38. **Stoll, B. J., R. I. Glass, M. I. Hug, M. U. Khan, H. Banu, and J. Holt.** 1982. Epidemiologic and clinical features of patients infected with *Shigella* who attended a diarrheal diseases hospital in Bangladesh. J. Infect. Dis. **146:**177–183.
39. **Svennerholm, A.-M., L. A. Hanson, J. Holmgren, B. S. Lindblad, B. Nilsson, and F. Quereshi.** 1980. Different secretory immunoglobulin A antibody responses to cholera vaccination in Swedish and Pakistani women. Infect. Immun. **30:**127–430.
40. **Svennerholm, A.-M., J. Holmgren, L. A. Hanson, B. S. Lindblad, F. Quereshi, and R. J. Rahimtoola.** 1977. Boosting of secretory IgA antibody responses in man by parenteral cholera vaccination. Scand. J. Immunol. **6:**1345–1349.
41. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharide extraction with phenol:water and further application of the procedure. Methods Carbohydr. Chem. **5:**83–91.